Int.J.Curr.Microbiol.App.Sci (2019) *8*(8): 1074-1080

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 8 Number 08 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.808.125

Detection of *E. coli* from Sheep and Goat Faecal Samples and Identification by Conventional and Molecular Assay

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Keywords

E. coli, Sheep, Goats, Biochemical test, PCR

Article Info

Accepted:
10 July 2019
Available Online:
10 August 2019

A B S T R A C T

E. coli is a Gram negative bacteria belonging to enterobacteriacea family. They are commonly found in the intestine of animals and humans. Most *E. coli* are harmless but some can cause infection. In our study, we isolated *E.coli* from 100 (50 from sheep and 50 from goats) faecal samples of diarrhoeic as well as healthy sheep. Isolation was done on BHI, MLA and EMB agar. The isolated bacteria suspected for *E. coli* were subjected to biochemical tests viz catalase, oxidase and IMViC. The isolates confirmed by biochemical tests were further confirmed by genus specific PCR. Out of 100 samples, 40 samples were positive for presence of *E. coli*. On BHI creamy mucoid colonies were formed, while as on MLA lactose fermenting pink colonies were formed. On EMB, colonies with characteristic metallic sheen were formed. All the isolates were catalase positive, oxidase negative and gave a characteristic pattern in IMViC test i.e. indole positive, methyl red positive, Voges Prausker and citrate negative. The 40 isolates were subjected to genus specific PCR out of which 26 isolates were confirmed as *E. coli*.

Introduction

E. coli is a Gram-negative, facultative anaerobe and nonsporulating bacterium. Cells are typically rod-shaped, and are about 2.0 µm long and 0.25–1.0 µm in diameter, with a cell volume of 0.6–0.7 μm^3 (Loo *et al.*, 2014). *Escherichia coli* is a bacterial commensal of the intestinal microflora of a variety of animals, including humans. Not all *E. coli* strains are harmless, as some are able to cause *diseases* in humans as well as in mammals and birds (Dho-Moulin *et al.*, 1999). Pathogenic *E. coli* strains fall into two categories: those that cause intestinal pathologies and those that cause extra intestinal pathologies. Intestinal pathologies mostly consist of more or less severe diarrhoea caused by different *E. coli* pathotypes such as enterotoxinogenic,

enteropathogenic or enterohaemorragic *E. coli* (ETEC, EPEC and EHEC, respectively), potentially evolving into a haemolytic uremic syndrome (HUS) in the case of EHEC infections (Kaper *et al.,* 2005). The emergence of verotoxigenic *E. coli* (VTEC) as human pathogens, contamination of food of animal origin has been a major public health concern. Infected cattle and sheep are considered the main reservoir of VTEC, with spread in human and animals associated with the consumption of contaminated beef and beef products (Griffin and Tauxe 1991) or direct contact with animals and animal faeces on the farm Around 29 strains of *E.coli* have been reported from sheep and 16 stains from goat (Vicente *et al.,* 2005). Animal and human commensal microbiota, especially intestinal microbiota, are subjected to numerous antimicrobial pressures due to farming practices and veterinary and human medicines. They have been suggested to play a major role in disseminating bacterial resistance (Kang *et al.,* 2005). Keeping these facts in view we isolated *E. coli* from faecal samples of sheep and goats which were confirmed by biochemical test and genus specific PCR.

Materials and Methods

Collection of samples and isolation of bacteria

The current study was ethically approved by Institutional Animal Ethics Committee. A total of 100 faecal samples were collected from healthy as well as diarrhoeic sheep and goats, from farms in and around Ludhiana, Punjab. These samples were aseptically collected in sample collection container and immediately kept in ice box and transferred to the laboratory. The samples were then subjected to bacterial isolation, followed by identification of isolates and PCR. The faecal samples brought to the laboratory were inoculated on BHI, MLA, EMB agar (Himedia). The inoculated plates were incubated at 37°C for 16-24 hours. The suspected colonies after incubation were subjected to Gram's staining for identification and subjected to various biochemical tests for confirmation.

Identification of isolates by biochemical tests

The isolates were confirmed by various biochemical tests like oxidase, catalase and IMViC test. IMViC test was performed using IMViC test kit (Himedia) as per the manufacturers' recommendations. Pick up the *E. coli* colonies from the media (EMB) by inoculation loop, then streak on to wells and incubated 37º C for 18- 24 hrs. For Indole test, after inoculation and incubation add 2-3 drops of Kovac's reagent in first well. If pink colour is obtained, then it is positive reaction. For MR test, add 1-2 drops of Methyl Red reagent in second well. If red colour obtained, the test is positive. For VP test, add 2-3 drops of Baritt reagent A and 1 drop of Baritt reagent B in third well. Pinkish red colour obtained 5-10 minutes indicates a positive test. For citrate test if colour change to blue then it is positive for citrate utilization.

Confirmation by genus specific PCR

Extraction of genomic DNA

The DNA of *E. coli* isolates was extracted using NucleoSpin® Microbial DNA kit (Macherey Nagel) as per manufacturer's instructions. Bacterial cells grown in a broth were harvested by centrifugation in a microcentrifuge tube and supernatant was discarded. Then approximately 40 mg of wet weight microbial cell culture pellet was taken and 100 µL Elution Buffer BE was added and cells were resuspended. Cell suspension was transferred into the NucleoSpin® Bead Tube Type B and 40 µL Buffer MG was added. Then, 10 µL Liquid Proteinase K was added

and mixed by vortexing for 4 min. NucleoSpin® Bead Tubes were centrifuged at 11,000 x g for 30 s. After this 600 µL Buffer MG was added, mixed and vortexed for 3 s. The tubes were centrifuged again for 30 s at 11,000 x g. Supernatant was Transferred (500–600 µL) onto the NucleoSpin® Microbial DNA Column, placed in a 2 mL Collection Tube. Again centrifugation was done for 30 s at 11,000 x g. Columns were transferred into a fresh Collection Tube. 500µL Buffer BW was added. Again the centrifugation was done for 30s at 11,000xg. After Discarding the flow through the columns were placed back into the Collection Tube and 500 µL Buffer B5 was added to the column and centrifuged for 30 s at 11,000 x g. In order to Dry silica membrane the column was centrifuged for 30 s at 11,000 x g. Then highly pure DNA was eluted by Placing the NucleoSpin® Microbial DNA Column into a 1.5 mL nuclease-free tube and 100 µL Elution Buffer BE was added to the column. The column was incubated was at room temperature for 1 min then centrifuged at 11,000 x g for 30 s. Purity and concentration of the extracted genomic DNA was estimated by using a Nanodrop (Thermo Scientific, USA).

Extraction of genomic DNA by hot-cold lysis method

Four to five colonies of the suspected *E. coli* isolates were taken into 1ml of sterile distilled water/ normal saline in 1.5 ml microcentrifugation tube (MCT) to make a homogeneous suspension. MCT containing the suspension was then placed into a water-bath preheated to 100°C. The suspension was boiled for 10 minutes at this temperature. MCT containing the suspension was then immediately transferred to ice kept at -20°C.Centrifugation was done and supernatant was collected. Extracted DNA was then stored at -20°C for further use.

Polymerase Chain Reaction (PCR)

PCR was done by using already published *E. coli* genus specific primers (Riffin *et al.*, 2001). A Reaction mixture of 25 µL was prepared consisting of 12.5 µL mastermix (Promega), forward primer 1 µL (20 pmol), reverse primer 1 µL(20 pmol), 3 µL of template DNA, 7.5 µL nuclease free water. PCR cycling conditions consisted of an initial denaturation at 94°C for 5 minutes followed 30 cycles each of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 1 minute.

This was followed by a final extension at 72°C for 10 minutes. The amplified products were analysed by electrophoresis at 80V for 1 hr in 1.0 % agarose gel in 1x TBE buffer containing ethidium bromide and visualized under Alpha imager gel documentation system (Alpha innotech) and photographed.

Results and Discussion

Isolation and identification of *E. coli* spp.

A total of 100 samples were processed for isolation of *E. coli*. Out of 100 samples 40 samples were positive for presence of *E. coli*. On BHI colonies are spherical, creamy mucoid in nature (Fig. 1), on MLA lactose fermented pink colour colonies were present (Fig. 2) and on EMB colonies shows metallic sheen (Fig. 3). Pink colour coccobacilli bacterial were present while doing Grams staining (Fig. 4). During confirmation by biochemical tests, *E.coli* were found to be catalase positive (Fig. 5), oxidase negative and in IMViC test indole, methyl red positive and Voges Proskauer and citrate negative (Fig. 6). The colony characteristics of the isolated *E. coli* in different media resemble the colony characteristics of *E. coli* as stated by Ali *et al.*, (1998). They reported that the faecal isolates showed various colony characteristics and

biochemical reactions in different bacteriological agar media. All the *E. coli* isolates were found to be positive in catalase, methyl-red positive and indole but negative to VP test which supports the findings of Beutin *et al.*, (1993). In the present study the isolated *E. coli* organism fermented dextrose, maltose, lactose, sucrose and manitol with the production of both acid and gas. Results of MR, Indole test of the *E. coli* isolates were positive as reported by Buxton and Fraser 1977. In Gram's staining, the morphology of the isolated bacteria exhibited pink, small rod shape, Gram negative bacilli which were supported by several authors (Buxton and Fraser 1977; Freeman 1985).

Fig.1 *E. coli* on BHI shows cream colour, mucus type colony

Fig.2 *E. coli* on MLA showing pink coloured colonies

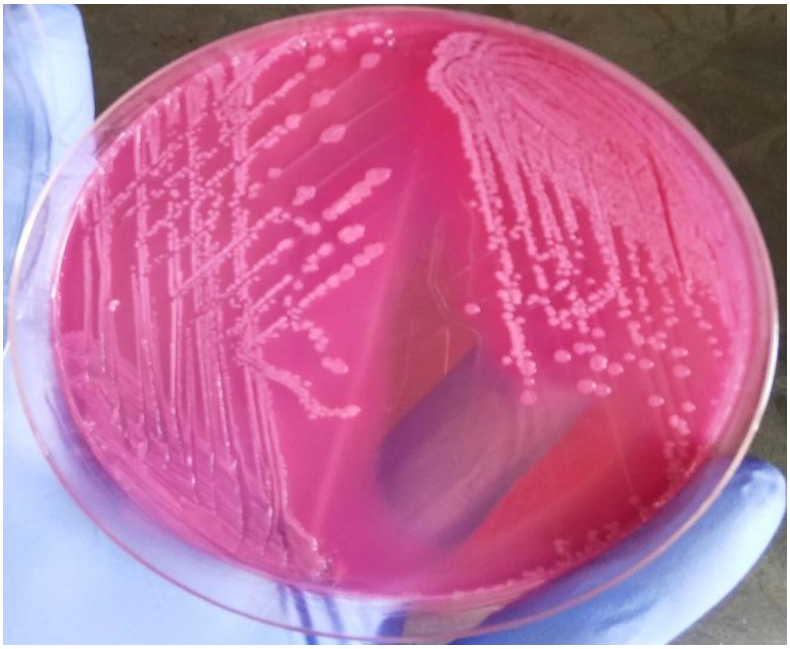


Fig.3 *E. coli* on EMB showing metallic sheen

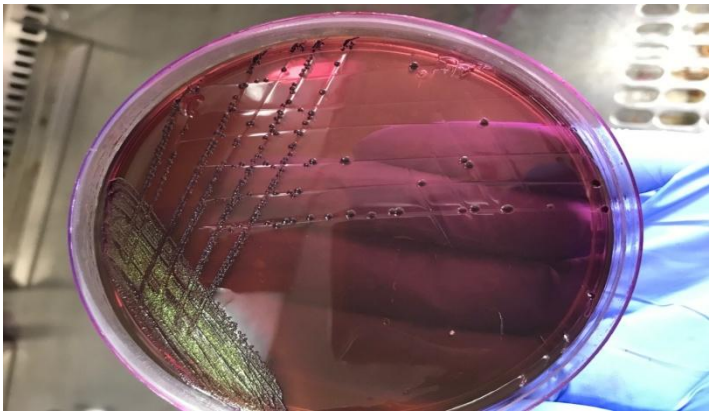


Fig.4 *E. coli* Gram negative bacteria shows pink colour bacilli

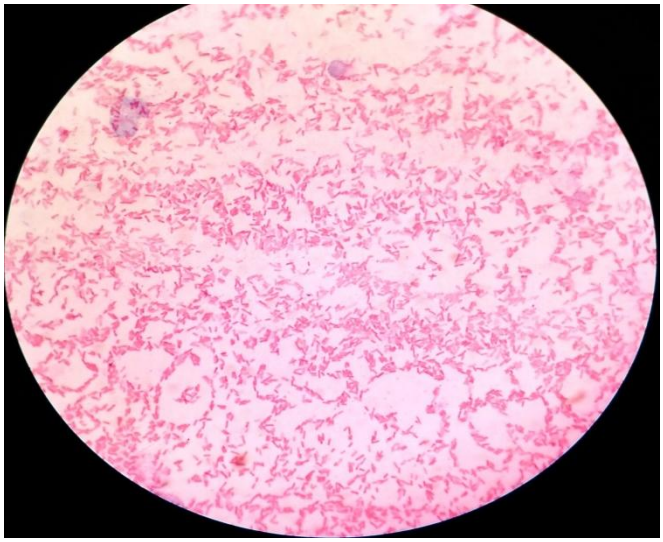
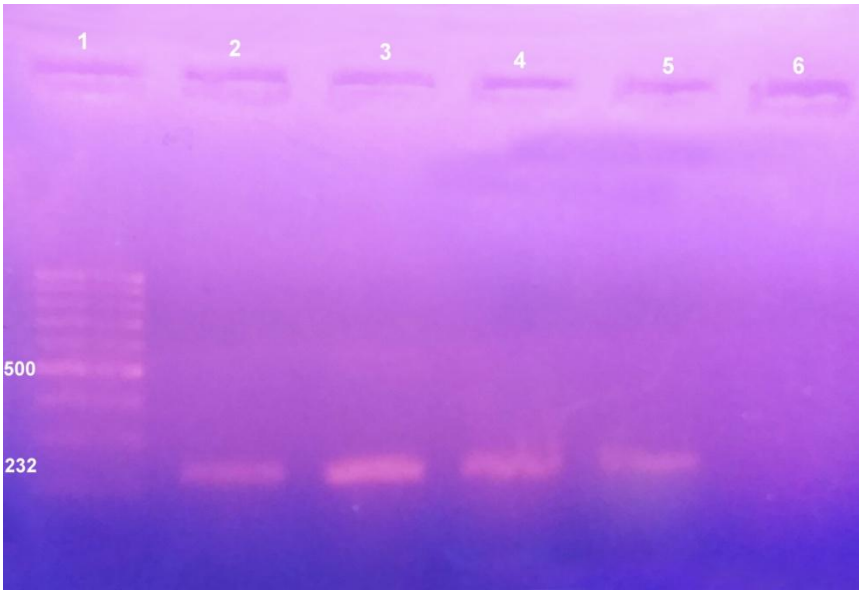


Fig.5 *E. coli* is catalase positive

Fig.6 Gel electrophoresis of PCR amplified fragment from *E.coli* isolates by using genus specific primer pair (L1=100 bp DNA ladder, L 2, 3, 4, = positive samples L=5 positive control and L= 6 negative control)



Confirmation by PCR

Out of the 40 isolates confirmed positive by biochemical tests 26 isolates were found positive by genus specific PCR. PCR gave an amplification of 232 base pairs (Fig. 6) when observed under U.V in gel doc which confirmed the *E. coli*.

Similar study was done by (Rekha *et al.*, 2006) and they confirmed *E. coli* isolates by conducting the genus specific PCR targeting *Mal* B promoter gene (585 bp).

Sabat *et al.*, (1999) conducted genus specific PCR for *E. coli* isolates targeting 16S r RNA gene of 544 bp. Nguyen *et al.*, (2016) confirmed the *E.coli* isolates by genus specific PCR targeting 16S r RNA gene of 654bp.

Out of 100 samples processed, 40 suspected *E.coli* isolates were identified on the basis of cultural characteristics and biochemical tests and out of 40 isolates, 26 were identified through genus specific PCR.

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How to cite this article:

Kumar, K., N.S. Sharma, Paviter Kaur and Arora, A.K. 2019. Detection of *E. coli* from Sheep and Goat Faecal Samples and Identification by Conventional and Molecular Assay. *Int.J.Curr.Microbiol.App.Sci.* 8(08): 1074-1080. doi: https://doi.org/10.20546/ijcmas.2019.808.125